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Analysis of selected pesticides and alkylphenols in human cord blood by gas chromatograph-mass spectrometer

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Abstract

A total of seven pesticides and eight alkylphenols were monitored using this method for the determination of their trace levels in human cord blood. The pesticides are lindane, diazinon, α-endosulfan, β-endosulfan, endosulfan sulfate, chlorpyrifos and endrin; while the alkylphenols are 4-*n*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol, 4-*t*-octylphenol, 4-*n*-heptylphenol, nonylphenol, 4-*n*-octylphenol and bisphenol A. The pesticides and alkylphenols in the cord blood samples were extracted with solid phase extraction IST C18 cartridges and analyzed by selected ion monitoring mode using quadrapole detector in Shimadzu QP-5000 gas chromatograph-mass spectrometer. Trace levels of pesticide and alkylphenols in the range of non-detectable to 15.17 ng ml^{-1}, were detected in the human cord blood samples. This technique of monitoring the levels of endocrine-disruptors in blood samples is consistent, reliable and cost effective while reducing wastage of time and solvents.

Keywords: Pesticide; Alkylphenol; Endocrine-disrupting chemicals; Human cord blood; GCMS

1. Introduction

Some pesticides and alkylphenols have endocrine-disrupting chemicals (EDCs) effects that are able to disrupt the chemical messengers system in the body. For the general population, the greatest exposure to EDCs is from food intake. Exposure may also come from pesticide residues remaining in fruits and vegetables and from drinking water [1]. Since many of the chemicals are soluble in fat, the highest levels are found in meat, fish and dairy product. It has been estimated for the persistent organochlorine chemicals for example, that about 80% of our intake of these chemicals come from these foodstuffs [2]. Some individuals may also be exposed to EDCs as a result of handling chemicals at work.

The developing young are directly exposed to EDCs that are circulating in the maternal body and may be passed directly to the eggs of egg laying animals, or through the placenta to the developing fetus in the womb of mammals and through breast milk to the nursing young [3]. The placenta is an effective barrier against fetal exposure to proteins or foreign bodies that might cause harm. It probably also protects the develop-

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ing embryo against hormones circulating in the maternal blood that might adversely affect its development [4]. Thus, the placental barrier is impervious to most sex hormones, including estrogen. However there are studies that show that EDCs such as bisphenol A easily crosses the placental barrier and enters the fetus [4,5]. It is thought that the body fat is mobilized before pregnancy and lactation, releasing persistent chemicals stored in fat [6]. This means that a proportion of chemicals that have accumulated in a woman's body during her whole lifetime may be passed to her child. In addition, other effects include increased cancer incidences, disrupting development of the immune and nervous system, early onset of puberty in girls and the population decline in human sperm count [7–10].

In this study, a method was developed to analyze trace levels of seven pesticides and eight alkylphenols in human plasma using solid phase extraction (SPE) and gas chromatograph-mass spectrometer (GCMS). The pesticides are lindane, diazinon, α-endosulfan, β-endosulfan, endosulfan sulfate, chlorpyrifos and endrin; while the alkylphenols are 4-*n*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol, 4-*t*-octylphenol, 4-*n*-heptylphenol, nonylphenol, 4-*n*-octylphenol and bisphenol A. Little is known about the possible effects of EDCs in humans, partly because of the unacceptability of doing deliberate experiments on humans. However, there are some worrying trends in the health of both men and women.

2. Experimental

2.1. Ethics approval and sample collection

The Ethics Committee of the University Malaya Medical Centre (UMMC), Malaysia approved the study of contaminants in human umbilical cord blood and collection of the umbilical cord blood upon delivery at labor ward at the UMMC, Malaysia. One hundred and eighty human umbilical cord blood samples were tested for the presence of alkylphenols and pesticides. Before delivery, the expectant mother was asked a series of questions about their pregnancy and their personal particulars were recorded. The cord blood was collected in a 6-ml glass lithium heparin Vacutainer (obtained from Becton Dickinson Vacutainer Systems, New Jersey, USA) upon delivery at the UMMC, Malaysia; and centrifuged at 3500 rpm for 10 min. The plasma supernatant was aspirated out and stored in a 14-ml glass vial at −20 °C.

2.2. Solvents and reagents

All alkylphenol standards and bis(trimethylsilyl)tri-fluroacetamide (BSTFA) were obtained from Wako Pure Chemical Industries, Ltd, Osaka, Japan. Naphthalene-d_8, phenanthrene-d_{10} and pyrene-d_{10} were obtained from Cambridge Isotope Laboratories, Inc., Massachusetts, USA. Pesticide standards were obtained from Riedel-de Haën Laborchemikalien GmbH & Co., Seelze, Germany. Solvents used for extraction and reconstitution such as methanol, dichloromethane and ethyl acetate were obtained from Fisher Scientific UK Ltd, Leicester, UK and are of HPLC grade. Phosphate buffer (0.04 M, pH 2) was prepared with deionized water. Sodium dihydrogen orthophosphate (NaH_2PO_4) used in preparing the phosphate buffer was obtained from BDH Ltd, Poole, England; while phosphoric acid, used to acidify the buffer, was obtained from Fisher Scientific UK Ltd, Leicester, UK.

2.3. Apparatus

SPE cartridges used for the extraction of alkylphenols and pesticides was ISOLUTE non-polar C18 sorbent (100 mg), obtained from International Sorbent Technology (IST) Ltd, Mid Glamorgan, UK. All quantitation of alkylphenols and pesticides were done with a Shimadzu QP-5000 GCMS and analyzed by selected ion monitoring mode using quadrapole detector. The GCMS column used for this analysis was a DB-1 column with a length of 30 m, internal diameter of 0.32 mm and column thickness of 0.25 μm manufactured by J & W Scientific, California, USA.

2.4. GCMS conditions

For the analysis of alkylphenols and pesticides, two different GCMS conditions were used. The GCMS column parameters for the alkylphenols analysis were as follows: initial temperature, 50 °C, hold for 2 min; then increased at 20 °C min^{-1} to 100 °C, then at 10 °C min^{-1} to 200 °C; and finally at 20 °C min^{-1} to 300 °C. Injector port temperature was set at 300 °C while the interface temperature was set at 270 °C. Column pressure program was initially at 40 kPa for 5 min, and then increased at 2 kPa min^{-1} to 70 kPa. Ionsets and retention time for the analysis of alkylphenols are shown in Table 1.

The GCMS column parameters for the pesticides analysis were as follows: initial temperature, 70 °C, hold for 2 min; then increased at 20 °C min^{-1} to 130 °C, and 5 °C min^{-1} to 200 °C; finally at 15 °C min^{-1} to 300 °C. Column pressure was at 50 kPa throughout the analysis. Injector port temperature and interface temperature were both set at 280 °C. Ionsets and retention time for the detection of pesticides are shown in Table 2.

2.5. Extraction method

This extraction method involves the use of IST ISOLUTE C18 SPE cartridge and the vacuum manifold station (IST VacMaster). The cartridge was first preconditioned with 2 ml of methanol followed by 2 ml of phosphate buffer (0.04 M, pH 2). 1 ml of plasma was passed through the cartridge at a rate of 1 ml min^{-1} followed by 2 ml of phosphate buffer (0.04 M, pH 2). The cartridge was then dried with vacuum suction for 10 min. The analytes were eluted out and collected using 3 ml of dichloromethane:ethyl acetate (1:1). The elution solvent was then completely dried under nitrogen stream.

Several blank samples were also prepared during the extraction of the patient samples, and follow the same extraction method. These blank samples were analyzed and found to be clear of any background levels of any of the chemicals.

Table 1
The ionsets and retention time for the alkylphenol analysis using GCMS

Alkylphenol	Ionsets	Retention time (min)
4-*n*-Butylphenol	179.0, 222.0	12.15
4-*n*-Pentylphenol	179.0, 236.0	13.40
4-*n*-Hexylphenol	179.0, 250.1	14.63
4-*t*-Octylphenol	207.0, 278.2	14.87
4-*n*-Heptylphenol	179.0, 264.0	15.63
Nonylphenol	207.0, 221.0, 193.0	15.84
4-*n*-Octylphenol	179.0, 278.2	16.50
Bisphenol A	357.2, 272.2	19.05
Internal standard		
Naphthalene-d_8	136.0	8.88
Phenanthrene-d_{10}	188.0	16.00
Pyrene-d_{10}	212.1	18.47

Table 2
The ionsets and retention time for the pesticide analysis using GCMS

Pesticide	Ionsets	Retention time (min)
Lindane	217, 219	13.33
Diazinon	304, 276, 227, 248	14.61
Chlorpyrifos	316, 314	17.96
α-Endosulfan	237, 269, 263	20.18
Endrin	263, 277, 248, 261	21.28
β-Endosulfan	263, 277, 237	21.35
Endosulfan sulfate	272, 274	22.25

2.6. GCMS quantitation

The analytes were reconstituted with 70 μl of dichloromethane:ethyl acetate (1:1), 20 μl of BSTFA (derivatizing agent) and 10 μl of internal standards (mixture of phenanthrene-d_{10}, naphthalene-d_8 and pyrene-d_{10}; concentration of each internal standard was 1 μg ml^{-1}). BSTFA attacks the –OH group of the alkylphenol and the result is a volatile trimethylsilyl derivative, thus making it easily detected in the GCMS. The internal standards were used to eliminate injection error while maintaining a constant area ratio for concentration quantitation. Naphthalene-d_8 acts as the internal standard for 4-*t*-butylphenol, 2,4-dichlorophenol and 4-*n*-butylphenol. Phenanthrene-d_{10} acts as the internal standard for 4-*n*-pentylphenol, 4-*n*-hexylphenol, 4-*t*-octylphenol, 4-*n*-heptylphenol, 4-nonylphenol, 4-*n*-octylphenol and penta-

chlorophenol. Pyrene-d_{10} acts as the internal standard for bisphenol A. One microliter of sample was injected into the Shimadzu QP-5000 GCMS.

Quantitation of the pesticides and alkylphenols in the sample were based on the comparison of area ratio of the compounds and internal standards to that of the compound standard calibration curves.

2.7. Preparation of calibration curve

Standard concentrations ranging from 0 to 50 ng ml^{-1} of pure alkylphenols and pesticides were prepared by using the serial dilution method. The concentrations were 50.0, 25.0, 12.50, 6.25, 3.13, 1.52 and 0 ng ml^{-1}. These concentrations were then injected into the GCMS and the calibration curves were calculated and drawn.

2.8. Method validation

Recoveries of most alkylphenols and pesticides extracted were in the range of 65–120% and the coefficients of variation (CV) of the compounds recovered were below 15%. There was an exception for nonylphenol, which had recoveries of above 200% across the complete calibration curve; but the high recoveries were constant and had CVs below 15%. The high recoveries could be due to disturbance of the nonylphenol detection peak with other impurities found in the serum which has the same retention time or a few similar ionsets as nonylphenol. Intraday and interday extractions showed consistent recoveries; CVs were observed to be below 15%. The intraday and interday recoveries showed that this SPE extraction method has high precision and consistent.

The limit of detection for the alkylphenols analysis was 0.05 ng ml^{-1} while for the pesticides analysis was 0.10 ng ml^{-1}. The limit of quantification for the GCMS alkylphenols analysis was 0.10 ng ml^{-1} while for the pesticides analysis was 0.25 ng ml^{-1}.

3. Results and discussion

The alkylphenols that were detected in the cord blood samples were 4-*n*-butylphenol, 4-hexylphenol, 4-*t*-octylphenol, nonylphenol, 4-*n*-octylphenol and bisphenol A while the only pesticide that was detected in the cord blood samples was chlorpyrifos. Nonylphenol was recorded as the highest level of EDCs detected in most cord blood samples followed by bisphenol A. The range of EDCs detected was as follows: nonylphenol (non-detectable—15.17 ng ml^{-1}), bisphenol A (non-detectable—4.05 ng ml^{-1}), 4-*n*-octylphenol (non-detectable—4.17 ng ml^{-1}), chlorpyrifos (non-detectable—1.84 ng ml^{-1}), 4-*t*-octylphenol (non-detectable—1.15 ng ml^{-1}), 4-*n*-butylphenol (non-detectable—0.80 ng ml^{-1}) and 4-*n*-hexylphenol (non-detectable—0.54 ng ml^{-1}). The percentage and number of samples with a positive detection of a particular alkylphenol or pesticide are shown in Table 3. Bisphenol A and nonylphenol were present in more than 80% of the samples, followed by 4-*n*-octylphenol (53%). The other compounds detected were lower than 20% of the sample size.

The range of total EDCs detected in the cord blood samples were divided into five groups and the breakdown of the compound concentration range is shown in Table 4 and Fig. 1. Approximately 90% of the cord blood samples were below 4 ng ml^{-1} of total EDCs detected and only a

Table 3
Samples with positive detection of the EDCs monitored

Compound	Number of samples with positive detection	Percentage of samples with positive detection (%)
4-*n*-Butylphenol	8	4
4-*n*-Hexylphenol	8	4
4-*t*-Octylphenol	31	17
Nonylphenol	155	86
4-*n*-Octylphenol	95	53
Bisphenol A	158	88
Chlorpyrifos	32	18

Table 4
Range of total EDCs present in the 180 human cord blood samples

Range of total EDCs present (ng ml^{-1})	Number of samples	Percentage of samples (%)
<2	92	51.11
2–4	68	37.78
4–6	15	8.33
6–8	3	1.67
>8	2	1.11

fraction of the cord blood samples had EDCs detection of above 8 ng ml^{-1}.

A Pearson's correlation test was performed between the levels of EDCs detected and a series of patient information taken during the interview before labor. Only selected patients particulars were taken for this test that was viewed could cause adverse health or could contribute to the levels of EDCs detected. No significant correlation was found between the levels of EDCs present in the blood when compared to the gestational period of the baby and birth weight. Frequency of eating raw vegetables in a week did give a near significant correlation when compared with the levels of EDCs in the cord blood but the frequency of eating cooked vegetables in a week did not show any significant correlation to levels of EDCs present in the cord blood.

There was also no correlation that supports the hypothesis of staying near an agricultural area or industrial area could lead to higher levels of EDCs in the human cord blood. The only way the mothers could be exposed to alkylphenols and pesticides is through their daily diet.

According to a study done by Guenther et al., they reported that nonylphenol is present in a wide variety of foods bought in German marketplaces, everything from gooseberry marmalade to liver sausages to chocolate crumble to double-cream cheese [11]. They speculated that a part of it could originate from nonylphenol ethoxylates which are used as non-ionic surfactants in disinfectants and cleaning agents or as emulsifiers in pesticides formulations. After application in food industries and agriculture, degradation of nonylphenol ethoxylates could lead to the accumulation of nonylphenol in food. In particular, the high concentrations of nonylphenol in apples and tomatoes could be consequently attributed to pesticide application. Then, the lipophilic nonylphenol would be accumulated in the wax coats of the fruits and vegetables, respectively. Another source might be plastic packaging materials from which nonylphenol, used for example in tris(nonylphenol)phosphate as antioxidant, could migrate into food [11].

Migration of 4-nonylphenol from polyvinyl chloride films for food packaging into food simulants has been studied in domestic applica-

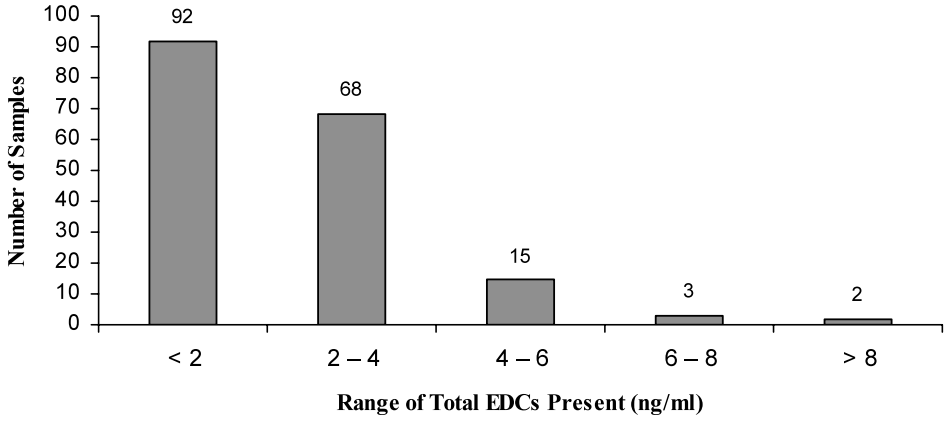

Fig. 1. Range of EDCs present in 180 cord blood samples.

tions such as wrapping of food and reheating in a microwave oven by Inoue et al. [12]. Another study done by Imanaka et al. observed the presence of bisphenol A in canned foods, packed food, fresh food such as fish and liver samples and box lunches found in Japan [13].

Fenske et al. observed in their study that most food samples in the daily diet of preschool aged 2–5 were exposed to detectable levels of organophosphorus pesticides such as chlorpyrifos, malathion, methidathion, methyl parathion and phosmet [14]. The fresh fruits and vegetable category had the most frequent pesticide determinations, followed by beverages. Cochran reported that chronic aggregate non-occupational exposures to chlorpyrifos ranged from 0.2 μg kg^{-1} per day (adult) to 3.0 μg kg^{-1} per day (infants and small children) [15].

Fan et al. observed nonylphenol could decrease the ability of spermatogenesis in rats without morphological changes of spermatogenic cells of the testes and epididymis [16]. They came to this conclusion in their study where pregnant rats were administered with *p*-nonylphenol during gestation and lactation at doses of 0, 50 100 and 200 mg kg^{-1} body weight, respectively. The weights of the testes and prostate of the young male rats of 70 days decreased with the increase doses of exposed nonylphenol. The same trend existed in daily production of sperm in the testes and sperm counts of the epididymis [16].

According to a study done by Takahashi and Oishi, absorption and distribution of bisphenol A in maternal organs and fetuses were extremely rapid and that placenta does not act as a barrier to bisphenol A [5]. Most bisphenol A absorbed by the intestine is probably glucuronidated exclusively in the liver and the conjugated form is excreted mainly into the bile after administering bisphenol A to rats [17].

Several studies have also shown that some alkylphenols and bisphenol A can primarily bioaccumulate in fat and affect energy balance [18,19]. 4-*t*-octylphenol does not bioaccumulate in rats receiving low oral doses, in agreement with the hypothesis of a rapid first-pass elimination of 4-*t*-octylphenol by the liver after oral ingestion, via glucuronidation and sulphation. Only if the detoxification pathways are saturated may excessive doses lead to bioaccumulation [19].

According to Slotkin et al., neonatal chlorpyrifos exposure produces widespread deficiencies in catecholaminergic synaptic function that persist in adulthood [20]. The effects caused by chlorpyrifos are likely to contribute to alterations in behavioral performance that persist or emerge long after the termination of chlorpyrifos exposure. Exposure to chlorpyrifos during developmental period in which this organophosphate pesticide is known to produce lasting changes in neural function, elicits corresponding, long-term deficits in immune competence [21].

4. Conclusions

The statistical evaluation of the cord blood results and past studies indicates that the ingested alkylphenols and pesticides can cross the placental barrier and reach a balance state between mother and baby. Although the potencies of the alkylphenols and pesticides to react as hormone agonists or antagonists are low compared to the natural ligands, the integrated response in the organism might be amplified by the ability of the xenobiotics to act via several mechanisms and the frequent simultaneous exposure to several xenobiotics. It is still uncertain whether these compounds at low concentrations will cause any adverse effects to the mother and child over a long exposure period since most of the toxicological studies administer dose levels of 100 times or greater to cause a positive effect.

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